

# Maternal and neonatal characteristics of pregnancies with detected thrombocytopenia and anti-HLA class I antibodies

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## **Abstract:**

*Introduction:* Anti-HPA 1a antibodies in connection with HPA 1a alloimmunized pregnancies have been shown to be associated with a lower birth weight in boys. The proposed mechanism for this was an affected placentation through binding of antibodies to the  $\beta_3$  integrin on vascular endothelial cells and/or invading trophoblasts. It was also hypothesized that thrombocytopenia could have affected placental vessel wall development. HLA class I antigens are also present on trophoblast cells and platelets, but it is not known whether anti-HLA class I antibodies could also affect foetal growth. *Methods:* Case control study. The case population (n=54) consisted of pregnancies with delivery of thrombocytopenic children with maternal anti-HLA class I antibodies. The control population (n=164) consisted of normal pregnancies with no clinical signs of bleeding or diagnosed thrombocytopenia. The control population was further divided into two groups: Those that tested positive for presence of anti-HLA class I antibodies in maternal samples (n=69), and those that tested negative (n=95). *Results:* We found significantly lower birth weight ( $p<0.001$ ), lower gestational age ( $p=0.001$ ), higher number of SGA children ( $p<0.001$ ), increased incidence of preeclampsia ( $p=0.026$ ), a higher placental weight/birth weight ratio ( $p<0.001$ ), increased proportion of nulliparous mothers ( $p<0.001$ ) and a higher mean optical density of anti-HLA class I antibodies ( $p<0.001$ ) in the case population compared with the antibody positive control population. In comparison, there were no significant differences in variables relevant for birth weight between the two parts of the control population. *Conclusion:* Our data suggests that the presence of anti-HLA class I antibodies might be associated with a reduced foetal growth in the context of thrombocytopenia in the newborn.

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## Introduction

The major histocompatibility complex (MHC) is a highly polymorphic cell surface molecule present in all jawed vertebrates[1]. It was first described in the mid-20th century by Gorer and Snell, by observations on how certain genes in mice resulted in resistance to allogenic tumor growth. The MHC in humans is called human leucocyte antigen (HLA), and is further differentiated into HLA class I and II molecules. The first HLA was identified by Jean Dausset in 1958 as a result of observations on how sera from several multitransfused patients agglutinated leucocytes from a high number of other individuals, while there was no agglutination of the patient's own leucocytes[2]. The first HLA, then called MAC, was later identified as HLA-A2[2]. Today there are 7527 described HLA alleles; 5880 of these are class I and 1647 are class II[3]. HLA class I is present on virtually all nucleated cells and platelets, and present peptides that have been processed from intracellular antigens. The class I alleles are divided into HLA-A, -B, -C, -E, -F and -G, as well as certain pseudoalleles. Of these, HLA-B is the most polymorphic, followed by HLA-A and HLA-C[3]. HLA class II is usually only present on professional antigen presenting cells (APC), such as dendritic cells, B-cells and macrophages, and present peptides processed from extracellular antigens, but may be present on all nucleated cells after activation[4]. The class II alleles are divided into HLA-DR, -DQ, -DP, -DM and -DO. Of these, HLA-DR is the most polymorphic[3].

### *T-cell activation*

The primary function of HLA molecules is the presentation of peptides processed from intra- or extracellular antigens to T cells. Intracellular antigens presented by HLA class I molecules can stimulate CD8<sup>+</sup> T cells, which subsequently can lead to lysis of the affected cell and further activation of the immune system. HLA class I molecules are also important as ligands for NK cells. Extracellular antigens presented by HLA class II molecules can stimulate CD4<sup>+</sup> T cells, which will then differentiate into T helper cells 1 or 2 (Th1 or Th2), depending on the cytokine environment. A Th1 response will primarily stimulate macrophage activity and proliferation of CD8<sup>+</sup> T cells, while a Th2 response will stimulate antibody production and class switch by proliferation of B cells and differentiation into plasma and memory cells. T cells can be activated by both self HLA (indirect allorecognition) as well as foreign HLA (direct allorecognition), as may be seen during transplantation.

### *Anti-HLA antibodies*

Antibodies directed against foreign HLA are most commonly observed in people that have been exposed to foreign HLA, such as multitransfused individuals or multiparous women. Most commonly these antibodies are directed against HLA class I molecules, as the class I molecule is much more abundant than class II. There are several complications associated with the presence of anti-HLA class I antibodies, such as rejection of allografts, destruction of transfused platelets and transfusion related acute lung injury (TRALI). The effect of anti-HLA class II antibodies is more disputed, but it has been shown an association between antibody-mediated rejection of allograft and the presence of anti-HLA class II antibodies[5].

### *Anti-HLA class I antibodies and pregnancy*

The prevalence of HLA class I antibodies in pregnant women is reported to be 7-39%[6]. The mechanism of alloimmunization is not completely understood, but it is known that foetal DNA and debris from trophoblast cells are present in the maternal circulation from about 7 weeks of gestation[7, 8]. Foetomaternal haemorrhage (FMH) is also a cause of alloimmunization, being the primary mechanism for haemolytic disease of the foetus or newborn (HDFN), in which a FMH can expose RhD-negative mothers to the D antigen.

Documented complications associated with the presence of HLA antibodies during pregnancies are few, but there have been reports of association between HLA immunization and recurrent abortions[9].

It is important to consider that HLA-C, but not HLA-A and -B, is expressed on fetal trophoblasts, along with HLA-E and -G[10, 11]. Of these three, only HLA-C express significant polymorphisms, and it has been shown that the interaction between HLA-C and uterine NK cells (uNK) in the decidua is important for placental development and function. uNK cells express killer-cell immunoglobulin-like receptors (KIRs) which binds to HLA class I. Most of these receptors induce inhibition of the killer cells when ligand is bound, but there are also activating receptors. KIR have two groups of haplotypes, A and B, which primarily differ in that B have additional activating receptors[12]. When considering HLA-C as a ligand for KIR, the different HLA-C allotypes can be grouped into C1 and C2 on the basis of dimorphism at position 80 of the alfa-1 domain[13, 14]. Both groups can act inhibitory or

activating depending on which KIR they bind to. As this interaction depends on two polymorphic gene systems there will be considerable variation from pregnancy to pregnancy, and certain combinations of KIRs and HLA-C allotypes have been shown to be associated with an increased risk of preeclampsia, foetal growth restriction and recurrent miscarriage[15-17].

CD8<sup>+</sup> cytotoxic cells, macrophages and dendritic cells are also affected by binding to both HLA-C and HLA-G on the trophoblasts. Finally, placental hormones also alter the function of maternal NK cells, regulatory T cells (T<sub>regs</sub>) and dendritic cells. Most of these interactions have an inhibitory effect on the immune response, and promote tolerance[7].

#### *Foetal/neonatal alloimmune thrombocytopenia and anti-HLA class I antibodies*

Foetal/neonatal alloimmune thrombocytopenia (FNAIT) is a potentially life threatening condition for the foetus or newborn, that is caused by incompatibility between foetal and maternal platelet antigens. Antigens present on the surface of foetal platelets but not on maternal platelets can lead to a maternal alloimmune response, in which IgG-antibodies produced by the mother can cross the placenta and lead to a destruction of foetal platelets. Depending on the severity of the destruction of the platelets, the resulting thrombocytopenia can lead to bleeding disorders in the foetus/newborn, where intracranial hemorrhage (ICH) is the most feared complication. However it should be noted that in most cases of FNAIT there are no clinical signs of bleeding.

FNAIT is defined as thrombocytopenia in the foetus or newborn caused by maternal alloimmunization. Thrombocytopenia is defined as a platelet count below  $150 \times 10^9 /L$ . Severe FNAIT is defined as a foetal platelet count below  $50 \times 10^9 /L$ [18]. FNAIT is found to occur in 1 per 1100 births[18], and is most commonly seen in children of mothers who are human platelet antigen (HPA)-1a negative, where the mother has been immunized by exposure to HPA-1a from the foetus. Several other HPAs have also been observed to cause FNAIT, and with these discoveries the number of antibody specificities being tested for in a suspected case of FNAIT is still increasing. But even with more extensive testing there are still several cases of suspected FNAIT where no antibody against an HPA is found.

Several case reports describe severe thrombocytopenia in otherwise healthy children, some with ICH, where the only finding from laboratory testing is a strong presence of anti-HLA class I antibodies[19, 20]. It is known that HLA-A, HLA-B and HLA-C are expressed on platelets, and that HLA class I incompatibility between mother and foetus can complicate foetal platelet transfusions [21] It has therefore been hypothesized that these antibodies also could cause FNAIT. A few small prospective studies have been performed, but an association between FNAIT and anti-HLA class I antibodies was not confirmed[6]. However, it should be noted that power is a problem with all prospective studies into FNAIT, due to the low incidence.

### *Antibodies and birth weight*

A recent study demonstrated a linear relation between the level of maternal anti-HPA 1a antibodies and birth weight in boys[22]. The same relationship could not be demonstrated in girls, but it is known that male sex is a risk factor in itself for adverse pregnancy outcome[23]. One proposed mechanism to explain why anti-HPA 1a antibodies could have an effect on birth weight, is related to the HPA 1 epitope on the  $\beta 3$  integrin, which is also expressed as part of the vitronectin receptor on vascular endothelial cells and invasive trophoblasts, playing an important role in angiogenesis and as an adhesion receptor for invading trophoblasts[24]. It was therefore suggested that antibodies directed against the HPA 1a antigen epitope on invasive trophoblasts could potentially disrupt placental vessel development and thus lead to placental impairment.

If this hypothesis is correct, one might argue that a similar mechanism might also apply to HLA antibodies. It is known that trophoblast invasion is defective in pregnancies that developed preeclampsia[25], and that placental insufficiency is the major cause of intrauterine growth restriction (IUGR) in the western world[26]. HLA-C, -E and -G is present on trophoblasts, and uNK cells, CD8<sup>+</sup> cytotoxic cells, macrophages and dendritic cells are affected by binding to both HLA-C and HLA-G. The effect of these ligations is mainly to down-regulate the immune response and promote tolerance. The binding of anti-HLA class I antibodies could possibly interfere with these ligations, and hence affect immune response and placentation.

IgG can cross the placenta due to the neonatal Fc-receptors on syncytiotrophoblasts. Anti-HLA class I antibodies may therefore enter the foetal circulation. It is still a matter of controversy whether maternal antibodies against paternal HLA class I may affect foetal or neonatal platelets, although anti-HLA class I antibodies are known to affect transfused thrombocytes in adults. If these antibodies do indeed affect foetal platelets, this could in theory also affect placental development, since it is known that platelets play an important regulatory role in angiogenesis[27]. One consequence could be reduced foetal growth with reduced birth weight in the neonate. In support of this, an increased occurrence of HLA antibodies in small for gestational age (SGA) infants with thrombocytopenia as compared to SGA infants without thrombocytopenia has been reported[28]. Small for gestational age is in Norway usually defined as birth weight below the 10th percentile for their gestational age[29].

## Aims

The aims of this paper were two-fold: First, to describe maternal and neonatal characteristics of pregnancies where anti-HLA class I antibodies were detected in pregnancy. Secondly, to compare maternal and neonatal characteristics of HLA class I alloimmunized pregnancies in general with HLA class I alloimmunized pregnancies where the newborn had thrombocytopenia, with special focus on fetal growth and birth weight.

## Methods

### *Study populations*

To investigate the possible effect of HLA class I antibodies on birth weight, two populations were selected: One population consisting of mothers who had delivered thrombocytopenic children, where the mother tested positive for anti-HLA class I antibodies postpartum and negative for platelet specific antibodies. The other population was selected from a group of non-selected healthy pregnancies where the child had no clinical sign of bleeding at the time of delivery. This group was then further divided into two groups: Mothers with detectable

anti-HLA class I antibodies at approximately week 24 of gestation, and mothers without detectable anti-HLA class I antibodies at approximately week 24 of gestation. A random selection of 45 children from the control group were also tested for platelet count at time of delivery using umbilical cord blood.

Our thrombocytopenic population, from hereon referred to as the case population, was selected from all suspected cases of FNAIT referred to the national reference laboratory for platelet immunology in Norway at UNN during 1998-2009. Inclusion criteriae were pregnancies where the neonate had thrombocytopenia at time of birth, maternal test for anti-HLA class I antibodies was positive and test for anti-HPA antibodies postpartum was negative upon analysis at the national reference laboratory, and that maternal post partum samples were available for further analysis. We excluded cases where there was an explanation for their thrombocytopenia other than a possible effect of antibodies. This included children born with syndromes and congenital infections that have been shown to cause thrombocytopenia in the newborn, and HELLP-syndrome (Hemolysis Elevated Liver enzymes Low Platelet count) in the mother. Mothers with only preeclampsia were not excluded, but additional analyses were conducted to evaluate their effect on the variables described.

Our non-thrombocytopenic population, from hereon referred to as the control population, are part of a non-selected population of pregnant women included over approximately the last 6 years in a prospective study into hemodynamic changes during pregnancy[30-34], conducted at University Hospital in North Norway (UNN). These women were selected on no other criteria than that they attended normal follow up of their pregnancy at the UNN, and that they accepted to be a part of the studies described. For this population maternal samples were collected at approximately week 24 of gestation, and clinical data registered in a separate database as part of the prospective study.

### *Laboratory analysis*

Anti-HPA antibodies were tested for using in-house monoclonal antibody immobilisation of platelet antigen (MAIPA) technique[35], modified after the procedure described by Kiefel et al.[36]. Maternal sample was tested against paternal thrombocytes or, if paternal



thrombocytes were not available, against thrombocytes from four random donors. An optical density value of 0.1 or more was considered positive.

Testing for anti-HLA class I antibodies in the control population were conducted using the same in-house modified MAIPA technique, with monoclonal mouse anti-human HLA-ABC antigen clone W6/32 (Dako Denmark A/S, Glostrup, Denmark) for immobilisation. Maternal samples were tested against thrombocytes from eight random donors. An optical density value of  $\geq 0.1$  was considered positive. If the analysis in MAIPA yielded a negative result, the maternal sera were also tested for anti-HLA class I antibodies using FlowPRA 1 Screening Test (One Lambda, Canoga Park, CA).

Testing for anti-HLA antibodies in the case population were conducted using the same in-house MAIPA protocol, with monoclonal mouse anti-human HLA-ABC antigen clone W6/32 (Dako Denmark A/S, Glostrup, Denmark) for immobilisation. Maternal sample was tested against paternal thrombocytes or, if paternal thrombocytes were not available, against thrombocytes from four random donors. An optical density value of  $\geq 0.1$  was considered positive.

#### *Placental weight versus birth weight ratio*

It has been shown that rather than to assess placental weight or birth weight separately own as indicators of more adverse outcome in newborn, they should rather be considered in correlation with one another as a placental weight versus birth weight ratio (PW/BW)[37]. A higher PW/BW ratio is associated with more adverse outcome. We used PW/BW when assessing foetal growth.

#### *Statistics*

All data were analyzed using SPSS software (version 19.0.0 SPSS inc.). Mean values and 95% confidence intervals were calculated for all continuous variables. Comparisons of interval variables were done using independent sample t-test. For comparisons of nominal variables a chi-square test was used when cell values were  $\geq 5$ , and Fisher's exact test was

used when one or more cell values were  $<5$ . A p-value of  $<0.05$  was considered statistically significant. We performed a linear regression analysis with birth weight as the dependent variable and study populations as the independent variable, where study populations consisted of two groups: Those that tested positive for anti-HLA class I antibodies in the control population, and the case population. In the regression analysis we adjusted for maternal age, parity, sex, placental weight, gestational age at delivery and preeclampsia

### *Ethics*

The project is part of a larger study approved by the Regional Ethical Committee (Regional etisk komite). Written consent was obtained from all participants.

### Results

Out of 462 suspected cases of FNAIT referred to the national reference laboratory for platelet immunology in Tromsø in the period 1998-2009, 83 cases (18%) matched our inclusion criteria. Of these 83 women, 63 (76%) agreed to participate. These 63 cases included 2 cases where the mother had given birth to twins. In one of these duplex pregnancies only one of the children was thrombocytopenic, and the other twin was therefore not included in the study population. Maternal sera were available in 58 of the 63 cases. Ten of the 64 children were excluded due to other plausible explanations for their thrombocytopenia: Two congenital cytomegalovirus (CMV) infections, two maternal HELLP-syndromes, one Jacobsen's syndrome, one maternal ITP during pregnancy, one neonatal hemochromatosis, one child where the thrombocytopenia first occurred one month after delivery, one Noonan's syndrome and one Down's syndrome. The final case population therefore included 54 cases.

Our control population consisted of 164 mothers, of which 69 (42.1%) tested positive for anti-HLA class I antibodies. Of these 69, 39 tested positive using MAIPA, while 30 were negative in MAIPA but positive when tested using FlowPRA 1 Screening Test. Those that tested positive for HLA class I antibodies in the control population will from hereon be referred to as the antibody positive control population, while those that tested negative will be referred to as the antibody negative control population.

Table 1: Description of the control population.

Variable	Antibody positive (n=69)	Antibody negative (n=95)	p-value
Age mother (95% CI)	30.5 years (29.3-31.6)	30.3 years (29.3-31.3)	0.841
Birth weight (95% CI)	3540 grams (3405-3676)	3480 grams (3335-3625) 3 missing	0.557
Gestational age at delivery (95% CI)	39.8 weeks (39.3-40.4)	39.8 weeks (39.3-40.4) 2 missing	0.975
Small for gestational age (%)	5 (7.2%)	6 (6.3%) 6 missing	1.000
Placental weight (95% CI)	627 grams (589-665) 5 missing	619 grams (590-648) 5 missing	0.745
Pw/Bw (95% CI)	0.178 (0.170-0.186) 5 missing	0.184 (0.174-0.194) 6 missing	0.403
Mode of delivery (%)	59 vaginal (85.5%) 7 sectio (10.1%) 3 missing	79 vaginal (83.2%) 9 sectio (9.5%) 7 missing	0.939
Preeclampsia (%)	1 (1.4%)	8 (8.4%) 4 missing	0.079
Sex (%)	34 boys (49.3%) 35 jenter (50.7%)	49 boys (51.6%) 41 girls (43.2%) 5 missing	0.518
Nullipara (%)	9 (13%)	40 (42.1%)	<0.001

*Antibody positive* signifies all cases where anti-HLA class 1 antibodies were detected, *antibody negative* signifies all cases that tested negative for presence of aHLA class 1 antibodies.

Table 2: Description of the case population as compared to the antibody positive control population.

Variable	Antibody positive control population (n=69)	Case population (n=54)	p-value
Age mother (95% CI)	30.5 years (29.3-31.6)	30.5 (29.1-31.9) 3 missing	0.981
Birth weight (95% CI)	3540 grams (3405-3676)	2954 (2663-3245) 2 missing	<0.001
Gestational age at delivery (95% CI)	39.8 weeks (39.3-40.4)	38.2 (37.2-39.1) 3 missing	0.001
Small for gestational age (%)	5 (7.2%)	23 (42.6%) 3 missing	<0.001
Placental weight (95% CI)	627 grams (589-665) 5 missing	597 grams (524-669) 22 missing	0.421
PW/BW ratio (95% CI)	0.178 (0.170-0.186) 5 missing	0.220 (0.197-0.244) 17 missing	<0.001
Optical density (95% CI)	1.03 (0.672-1.40)	2.30 (1.95-2.66) 3 missing	<0.001
Mode of delivery (%)	59 vaginal (85.5%) 7 sectio (10.1%) 3 missing	27 vaginal (50.0%) 25 sectio (46.3%) 2 missing	<0.001
Preeclampsia (%)	1 (1.4%)	5 (9.3%) 13 missing	0.026
Sex (%)	34 boys (49.3%) 35 girls (50.7%)	31 boys (57.4%) 21 girls (38.9%) 2 missing	0.259
Nullipara (%)	9 (13%)	23 (42.6%) 3 missing	<0.001

The mean maternal age in the case population was 30.5 years. In the control group the mean maternal age was 30.3 years for the antibody negative and 30.5 for the antibody positive, and this difference was not statistically significant ( $p=0.841$ , independent samples t-test). Out of the 164 mothers in the control population 49 were nulliparous (30%), 79 were primiparous (48%), and 36 were multiparous (22%). Out of the 54 mothers in the case population 23 were nulliparous (43%), 21 were primiparous (39%), and 7 were (13%) multiparous.

When comparing the two control populations, there was a statistically significant higher number of nulliparous women among the antibody positive ( $p < 0.001$ , chi-square test). There were also a statistically higher amount of nulliparous women in the case population compared to the antibody positive control population ( $p < 0.001$ , chi-square test). Data on gravida status was only available for the case population. Out of the 23 women who were nulliparous 11 (48%) were primigravida, 7 (30%) were multigravida and 5 (22%) had no data available. Out of the seven who were multigravida, five had only one earlier pregnancy.

There were significantly more mothers with preeclampsia in the case population compared to the antibody positive control population ( $p = 0.026$ , Fisher's exact test). There was no difference in number of mothers with preeclampsia between the two control populations ( $p = 0.079$ , Fisher's exact test).

There was no statistically significant difference in mean placental weight between either the two control populations ( $p = 0.745$ , independent sample t-test), or between the antibody positive control population and the case population ( $p = 0.421$ , independent sample t-test). There was however a statistically significant higher mean PW/BW ratio in the case population compared to the antibody positive control population ( $p < 0.001$ , independent sample t-test), with a mean PW/BW ratio of 0.178 in the antibody positive control population and 0.220 in the case population.

A significantly higher proportion of women in the case population was delivered by caesarean section compared to the antibody positive controls ( $p < 0.001$ , chi-square test). In the case population 46.3% had caesarean section as mode of delivery, where 16.0% were elective and 84.0% were acute. There was no statistically significant difference in mode of delivery between the two parts of the control population ( $p = 0.939$ , chi-square test).

Neonates in the case population were delivered significantly earlier than controls ( $p < 0.001$ , independent samples t-test). The mean gestational age at delivery was 39.8 weeks for both the antibody negative and the antibody positive control population. In the case population the mean gestational age at delivery was 38.2 weeks.

The mean birth weight for the case population was significantly lower than the antibody positive control population ( $p < 0.001$ , independent samples t-test). The mean birth weight in

the case population was 2954 grams. In the control population the mean birth weight was 3540 grams among the antibody positive, and 3480 grams among the antibody negative. There was no statistically significant difference in birth weight between the two parts of the control population ( $p=0.841$ , independent samples t-test).

There was a significantly higher number of SGA children in the case population compared to the antibody positive controls ( $p<0.001$ , chi-square test). There were 23 SGA children in the case population (42.6%). There were 11 SGA children in the control population (6.7%); 6 that tested negative for anti-HLA class I antibodies and 5 that tested positive.

There was no statistically significant difference in distribution of sex between any of the study population groups (Fig. 1 and 2). It is still noteworthy that the majority of the 23 SGA children in the case population were boys ( $n=16$ , 69.6%). In comparison, 2 of the 5 SGA children in the antibody positive control population were boys (40%), but this difference in gender distribution was not statistically significant ( $p=0.315$ , Fisher's exact test).

The mean nadir platelet count for the case population was  $30.8 \times 10^9/L$  (95% CI:  $25.0 - 36.6 \times 10^9/L$ ). Platelet counts were not available for all of the controls, but none were thrombocytopenic out of the 45 we randomly selected for analysis of umbilical cord blood (data not shown).

There was a statistically significant higher mean optical density of anti-HLA class I antibodies in the case population compared to the antibody positive control population ( $p<0.001$ , independent sample t-test).

Fig. 3: Description of the case population, when excluding all cases where preeclampsia in the mother was confirmed or the absence of it could not be confirmed, as compared to the antibody positive control population.

Variable	Antibody positive control population (n=69)	Case population excluding preeclampsia (n=36)	p-value
Age mother (95% CI)	30.5 years (29.3-31.6)	31.1 years (29.3-32.9)	0.539
Birth weight (95% CI)	3540 grams (3405-3676)	3141 grams (2782-3500)	0.011
Gestational age at delivery (95% CI)	39.8 weeks (39.3-40.4)	38.5 weeks (37.4-39.7)	0.020
Small for gestational age (%)	5 (7.2%)	11 (30.6%)	0.001
Placental weight (95% CI)	627 grams (589-665) 5 missing	634 grams (545-723) 7 missing	0.940
PW/BW ratio (95% CI)	0.178 (0.170-0.186) 5 missing	0.215 (0.189-0.241) 7 missing	0.001
Optical density (95% CI)	1.03 (0.672-1.40)	2.35 (1.94-2.75)	<0.001
Mode of delivery (%)	59 vaginal (85.5%) 7 sectio (10.1%) 3 missing	19 vaginal (52.8%) 17 sectio (47.2%)	<0.001
Sex (%)	34 boys (49.3%) 35 jenter (50.7%)	21 boys (58.3%) 15 girls (41.7%)	0.418
Nullipara (%)	9 (13%)	14 (38.9%)	0.003

*Case population excluding preeclampsia* signifies all cases in the case population except those where preeclampsia in the mother was confirmed or the absence of it could not be confirmed.

Regarding the SGA neonates in the case population, preeclampsia was present in 4 of the 23 (17%) pregnancies. In 8 further SGA pregnancies preeclampsia could not be ruled out. When all pregnancies with preeclampsia or where preeclampsia could not be ruled out were excluded, there were no differences in statistical significance in the variables already described.

In the linear regression analysis, neonates with thrombocytopenia weighed on average 238 grams lower compared with antibody positive controls after adjusting for confounding variables ( $p=0.014$ ). When excluding all cases where preeclampsia in the mother was confirmed or the absence of it could not be confirmed from the case population, and also not adjusting for preeclampsia, we found that the same analysis yielded a 230 grams ( $p=0.020$ ) lower birth weight among cases compared to antibody positive controls.

## Discussion

The aims for this study were two-fold: First, to describe maternal and neonatal characteristics of pregnancies where anti-HLA class I antibodies were detected in pregnancy. Secondly, to compare maternal and neonatal characteristics of HLA class I alloimmunized pregnancies in general with HLA class I alloimmunized pregnancies where the newborn had thrombocytopenia.

We found that 42% of our control group tested positive for presence of anti-HLA class I antibodies, which is in accordance with what has been previously reported of 7-39%[6]. The optical density was significantly higher among the case population as compared to the antibody positive control population ( $p < 0.001$ ), which would seem to indicate that there was a stronger presence of anti-HLA class I antibodies in our case population as compared to the antibody positive control population. However, it is important to note that optical density is only a semi-quantitative measurement of antibody level, and that this analysis does not say anything about the antibody specificities present.

The maternal and neonatal characteristics described in our control population were all in range of what would be expected from a normal population[38], and there was no statistically significant difference in characteristics between the two parts, except for the number of nulliparous women ( $p < 0.001$ ). It was to be expected that the amount of nulliparous women would be higher among those that tested negative for anti-HLA class I antibodies in the control population, as it has been described that the prevalence of HLA antibodies in pregnant women increases with number of pregnancies[39]. Parity is no precise description of number of pregnancies, but data on gravida status was only available for part of the case population. In the case population at least 48% of the nulliparous women were primigravida.

The maternal and neonatal characteristics of our case population showed a significant difference in multiple variables when compared to the antibody positive control population. The weight reduction in the case population was 238 grams compared with the antibody positive controls, which is comparable to the weight loss seen from active maternal smoking during pregnancy[40].

As we could only demonstrate a decreased birth weight in the case population and not in the antibody positive control population, it seems that the presence of anti-HLA class I antibodies in itself may not be the triggering factor regarding the more adverse outcome in our case population. An alternative explanation for the effect on birth weight could be the thrombocytopenia in itself, and its potential effect on foetal growth. It is known that thrombocytopenia affects angiogenesis[27]. And if this in turn affects placentation one would expect to see an altered foetal growth as a result, given that placental insufficiency is the major cause of intrauterine growth restriction (IUGR) in the western world[26].

There was a significantly higher number of mothers with preeclampsia in the case population as compared to the antibody positive control population, and this could suggest that placentation was affected in the case population. The PW/BW ratio was significantly higher in the case population compared to the antibody positive control population ( $p < 0.001$ ), and a higher PW/BW ratio is associated with more adverse outcome. This does not exclusively suggest that placentation was affected, although it remains a possibility. It should also be noted that none of the mean values for PW/BW ratios were above the 75th percentile or below the 10 percentile for expected ratios at the mean gestational age for the two groups, and hence within what has been described as the normal range[37].

If placentation was affected in the case population, it still would not seem to account for all the differing results between the case population and the antibody positive control population, as there were no changes in statistical significance when all pregnancies with preeclampsia or where preeclampsia could not be ruled out were excluded. Some sources have also disputed whether preeclampsia really is associated with a lowered birth weight[41].

When discussing our results it is important to consider the fact that nulliparity was also significantly higher in the case population as compared to the antibody positive control population. Nulliparous women have been shown to be at an increased risk of delivering a SGA child[42, 43], as well as at an increased risk of preterm birth[43]. But it has also been suggested that this association with at least preterm birth is affected by maternal age[44], which did not differ between the two groups who tested positive for presence of anti-HLA class I antibodies. Nulliparity might also have affected the presence of preeclampsia in our case population, as the risk of preeclampsia is higher among women under 20 years[45], and is generally considered a disease of first pregnancy[45].



If nulliparity is the reason why we found increased prevalence of preeclampsia, GA and number of SGA children in the case population, one would also expect this difference to be seen between the two groups of the control population, as the antibody negative part had a significantly higher number of nulliparous women than the positive part. This was not the case, as nulliparity was the only variable that showed any significant difference between the two groups. Therefore it seems likely that the differences between the case population and the antibody positive control population should be attributed to another factor. But it remains puzzling that a population selected based on the presence of anti-HLA class I antibodies should have such a high amount of nulliparous women, as the prevalence of anti-HLA class I antibodies in pregnant women have been shown to increase with number of pregnancies[39].

One could argue that perhaps the nulliparous women in the case population for some reason had trouble completing pregnancies, and that they therefore actually had been pregnant multiple times although they were nulliparous. However, only 30% of the nulliparous women in the case population were multigravida, and 70% of these had only had one earlier pregnancy.

Male gender is an independent risk factor for adverse pregnancy outcome[23]. Previously, the effect of anti-HPA 1a antibodies on birth weight could only be found in boys[22]. There were 49% boys in the antibody positive control population and 58% boys in the case population. The difference between these two groups was not statistically significant. Of the SGA children in the case population 70% were boys, which could indicate that whatever factor that contributed to the decreased birth weight had a more significant effect on the males, which is in accordance with for example what is observed on how male foetuses are more severely affected by maternal alloimmunization to the RhD and HPA-1a antigens[22, 46].

Our results seem to indicate that if anti-HLA class I antibodies have an effect on foetal growth, it would appear that these effects are in some way correlated with the presence of thrombocytopenia. It has not yet been demonstrated that maternal anti-HLA class I antibodies affect foetal or neonatal thrombocytes, and therefore it still remains a possibility that the effects seen in the case population are related to the thrombocytopenia alone, and not the presence of anti-HLA class I antibodies. Our results could thereby possibly be explained by another factor causing the thrombocytopenia, as for example an unidentified infection. This

does however remain unlikely given the size of our population, and the fact that extensive clinical history was available.

The possible effect of anti-HLA class I antibodies on foetal growth is supported by the fact that we found higher OD values in the case population as compared to the antibody positive control population. Although OD is only semi-quantitative, this does at least suggest that there was a stronger presence of antibodies in the case population, and could provide a possible explanation for the more adverse outcome in the case population. If the antibodies did affect foetal growth, it would be reasonable to assume that a stronger presence would provide a stronger effect.

It should also be considered that it is not completely known whether anti-HLA class I antibodies themselves cause complications in pregnancies, or if HLA class 1 alloimmunization more often occur as a result of pregnancy complications instead of being the cause of complications. Did the increased number of caesarean sections in our case population come as a result of the antibodies and their effects, or did the complications that potentially resulted in caesarean sections cause the production of antibodies? What is the hen and what is the egg here?

This study shows that a further analysis of the antibodies in our case and control populations is needed. Not only into whether there really was a stronger antibody presence in our case population, but also as to which antigen specificities were involved. If the characteristics of the maternal antibodies are significantly different between our case population and the control population, it might help to explain why a potential effect of anti-HLA class I antibodies on foetal growth was only seen in our case population, and not in the antibody positive control population.

## Conclusions

Our data suggests that the presence of anti-HLA class I antibodies might be associated with a reduced foetal growth in the context of thrombocytopenia in the newborn. There also seems to be a stronger presence of anti-HLA class 1 antibodies in the thrombocytopenic children

compared to the control population, but further studies are needed to quantify the antibody levels and determine antibody specificities.

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